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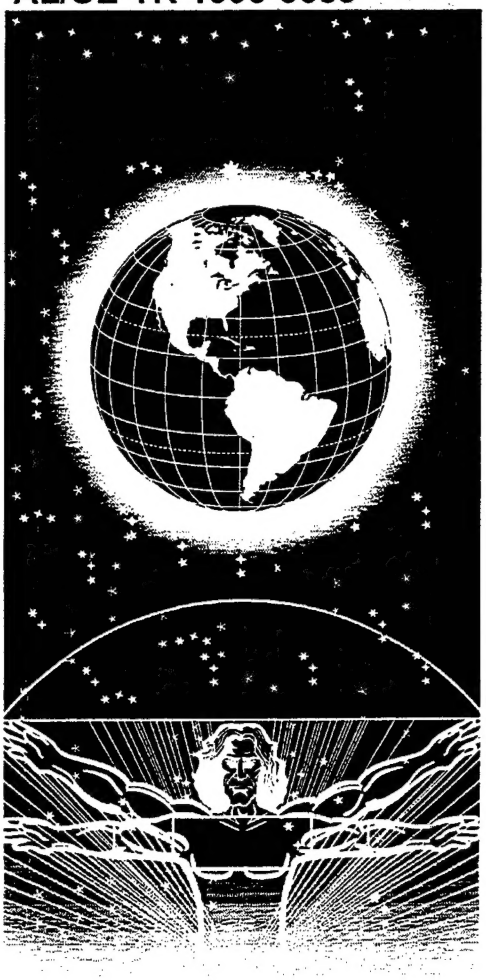

UNITED STATES AIR FORCE
ARMSTRONG LABORATORY

TRICHLOROETHYLENE AND CHLORAL HYDRATE METABOLISM IN THE JAPANESE MEDAKA MINNOW (ORYZIAS LATIPES) IN VITRO

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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

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FOR THE DIRECTOR

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The drive to assess more potential human health and environmental problems, coupled with decreasing budgetary resources has led to the investigation of alternative test methods. For an alternative method to be developed, relevancy to the target organism (in this case, the human) should be critically evaluated. The Japanese medaka (Oryzias latipes) is a small fish species which has some very distict andvantages: the species is small enough to be reared in large numbers under relatively simple environmental conditions, the gestation peroid is short so that many generations can be tested in a short peroid, its size enables the histological examination of multiple organ systems from a single prepared slide, its growth and develpment have been well characterized, and it appears to respond in a predictable manner to envirionmental carcinogens. However, in the assessment of compounds which are metabolized to be come toxic, the relevance of the medaka as a surrogate for the human has been less well-characterized. While some previous investigations have examined the soluble enzyme system, none to date have evaluated the cytochrome P-450 system, a system restricted to the endoplasmic reticulum of the cell. Because of the environmental prominence of trichloroethylene (TRI) and the role of metabolism in the toxicity of TRI, we sought to determine the capcity of the Japanese Medaka to metabolize this compound. In experiments conducted in vitro, we have determined that TRI is metabloized to the same metabloite (chloral hydrate, CH) as initially formed by the microsomal P-450 systems expressed in mammalian cells. Further, in medaka preparations, CH is metabloized to the same metablites as are produced by mammalian enzyme systems.

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PREFACE

The goal of this study was to determine whether the medaka minnow metabolizes trichloroethylene, a common groundwater contaminant, similar to metabolism in the rodent and human. This work was funded by the U. S. Army Biomedical Research and Development Laboratory, Fort Detrick, MD, Mr. Henry Gardner, Jr., Director. We gratefully acknowledge the contribution of technical training and medaka by Dr. Bill Hawkins, Mrs. Sue Barnes and other staff at the Gulf Coast Research Laboratory, Ocean Springs, MS; the contribution of medaka microsomes by Dr. Michael Miller and Mr. Craig Stamm of the West Virginia University Department of Biochemistry; and the demonstration of microsomal cytochrome P-450 proteins by Dr. Stelvio Bandiera of the Faculty of Pharmaceutical Sciences, University of British Columbia, Canada. The animals used in this study were handled in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council, DHHS, National Institute of Health Publication 86-23, 1985, and the Animal Welfare Act of 1966, as amended.

TABLE OF CONTENTS

SECTION	PAGE
PREFACE	iii
TABLE OF CONTENTS	iv
LIST OF TABLES	v
LIST OF FIGURES	vi
EXECUTIVE SUMMARY	1
INTRODUCTION	2
EXPERIMENTAL METHODS	4
Animals and Tissue Preparation	4
Exposure Conditions	4
Mass Spectral Confirmation of Chloral Hydrate	4
Quantification of Chloral Hydrate	5
Electrophoresis of Proteins and Western Blot Analyses	5
RESULTS	6
Demonstration of Chloral Hydrate as a Trichloroethylene Metabolite in Medaka Microsomes	8
Demonstration of Protein and Time Dependence of Chloral Hydrate Formation	8
Kinetics of Formation of Chloral Hydrate from Trichloroethylene	9
Evaluation of Sex-Differences in Rates of Biotransformation	10
Immunochemical Probing of Microsomes for Cytochrome P-450 Forms	12
DISCUSSION	14
CONCLUSION	16
REFERENCES	17

LIST OF TABLES

TABLE **PAGE**

1 Comparison of Selected Hepatic Metabolic Parameters Between Male and Female Medaka 10

2 Metabolism of Chloral Hydrate by Hepatic S_9 Fraction From Male and Female Medaka 12

LIST OF FIGURES

FIGURE		PAGE
1	Gas Chromatography/Selected Ion Monitoring Confirmation of Chloral Hydrate Formation from Trichloroethylene by Medaka Microsomes	7
2	Time and Protein Dependence of Trichloroethylene Metabolism	8
3	Kinetics of Chloral Hydrate Production from Trichloroethylene	9
4	Sex Dependent Metabolism of Trichloroethylene to Chloral Hydrate in Microsomes	11
5	Immunochemical Detection of CYP2E1 and CYP1A1 in Microsomes of Rats, Mice, Humans, and Medaka	13

EXECUTIVE SUMMARY

Trichloroethylene (TRI) is a common groundwater contaminant that has been shown to be tumorigenic and toxic in laboratory animals. The toxicity of TRI appears to be contingent upon the production of cytochrome P-450-dependent metabolites. Cytochrome P-450 2E1 metabolizes TRI in mammals; however, this isoform of cytochrome P-450 has not been reported to be expressed in the fish species examined to date. The objective of this study was to determine whether the Japanese medaka minnow (*Oryzias latipes*) metabolizes TRI to chloral hydrate (CH) and trichloroethanol (TCOH) in a manner similar to rats, mice and humans, thereby supporting the role of the medaka in risk assessments for TRI.

Livers were removed from euthanized male and female medaka and prepared by standard methods. The protein content of samples was determined using the BCA method and microsomal P-450 content was determined by carbon monoxide binding. Protein recovery data indicated a sex-specific distribution of total liver mass, total liver protein and S_9 (metabolically active) protein. Although the body mass of the adult female is approximately 71% that of the male, the female has more liver mass (18.93 mg; 5.35% body mass) per fish than the male (12.44 mg; 3.27% body mass), more S_9 protein (1.54 versus 0.88 mg per gram body mass), more cytochrome P-450 (0.38 versus 0.27 nmoles P-450 per gram body mass) and more activity towards an enzyme-marker substrate (ethoxyresorufin O-deethylase: 4.734 versus 3.674 pmoles/minute/gram liver). Activity towards dimethylnitrosamine (DMN), a marker for P-450 2E1 activity, was not detectable.

TRI was incubated with medaka microsomal protein and metabolites were extracted with ethyl acetate. The extracts were analyzed using gas chromatography (liquid injection) with an electron capture detector and separately using gas chromatography-mass spectrometry. We observed microsome-mediated metabolism of TRI to CH, a precursor of toxic metabolites. Linear relationships between the formation of CH and both exposure time and protein concentration were demonstrated. In addition, CH was incubated with medaka S_9 protein containing cytochrome P-450 and soluble enzymes was exposed to CH. Incubations were subjected to ethyl acetate extraction and a second method involving acidification then derivitization with dimethylsulfate followed by hexane extraction. Ethyl acetate and hexane extracts were analyzed by gas chromatography (liquid injection) with an electron capture detector. Both methods demonstrated the metabolism of CH to TCOH, although initial efforts failed to demonstrate trichloroacetic acid formation.

In a second series of experiments, samples of male and female microsomes were analyzed for individual cytochrome P-450 forms via polyacrylamide gel electrophoresis and immunoblotting with antibodies selective for individual cytochrome P-450 forms. These experiments confirmed expression of the cytochrome P-450 1A isoforms in the medaka, while cytochromes P-450 of the 3A and 2E families were not detected.

Together, these results are the first to indicate that medaka are capable of metabolizing TRI to CH and CH to TCOH. Metabolism of TRI in a species with no detectable DMN activity or 2E1 protein suggests that TRI is metabolized by P-450 forms other than 2E1. These data further support medaka's use in environmental and conventional risk assessments for this particular groundwater contaminant.

INTRODUCTION

Trichloroethylene (TRI) is an unsaturated, chlorinated hydrocarbon widely used as an industrial solvent. Because of its widespread usage it is also a relatively common water pollutant identified in many groundwater supplies (Murray and Riley, 1973). Exposure of the human population has also occurred through its industrial use and its use in obstetrical and dentistry anesthesia. Due to the documented and continued exposure of humans to TRI, toxicity of TRI has been evaluated in available animal models and in rodent carcinogenicity bioassays. These studies have yielded mixed results. When administered by gavage in corn oil, TRI produced increased hepatocellular carcinoma in $B6C3F_1$ mice, but had no such effect in Osborne-Mendel rats (NCI, 1976). TRI also produced nephrotoxicity in rats (Chakrabarti and Tuchweber, 1988; Goldsworthy *et al.*, 1988; Green and Odum, 1985; Maltoni and Cotti, 1986) and lung tumors and clara cell injury in mice (Fukuda *et al.*, 1983; Forkert *et al.*, 1985; Forkert and Birch, 1989; Lewis *et al.*, 1984; Maltoni and Cotti, 1986).

Differences in the toxicities expressed across species may be related to metabolism and pharmacokinetics (Green, 1990). The incidence of liver tumors is highest in the species (mice) which in general possesses the highest metabolic capacity for xenobiotics and which demonstrate higher rates of TRI metabolism *in vivo* (Green and Prout, 1985; Larson and Bull, 1992). The toxicity of TRI has been demonstrated to be a result of P450-dependent metabolism (Buben and O'Flaherty, 1985; Carlson, 1974; Moslen *et al.*, 1977b). Further, the induction of TRI metabolism in rodent species has been correlated with increased toxicity (Allemand *et al.*, 1978; Carlson, 1974; Cornish and Adefuin, 1966; Moslen *et al.*, 1977a,b; Nakajima *et al.*, 1988; Okino *et al.*, 1991). Nakajima and coworkers (Nakajima *et al.*, 1990; Sato and Nakajima, 1985) have demonstrated the effect of known P450 inducers (ethanol, phenobarbital and 3-methyl cholanthrene) on microsomal TRI metabolism. Microsomes from ethanol-treated animals possessed increased low-K_m TRI metabolism. Phenobarbital increased high-K_m TRI metabolism and microsomes from 3-methyl cholanthrene rats had an intermediate-K_m activity that was not increased by inducer-treatment (Nakajima *et al.*, 1988, 1990). These researchers have also examined the role of different P450 forms on TRI metabolism by examining the effect of form-specific antibodies on TRI metabolism. Antibodies to P450 2E1 and P450 1A2 inhibited CH formation to a greater extent in mice than rats; in rats, antibodies to P450 2C11/6 inhibited CH formation greater than in mice (Nakajima *et al.*, 1992). These data indicate that multiple P450 forms may catalyze TRI metabolism in the rodent.

At present, the validity of establishing a human health risk assessment for TRI based upon rodent toxicity data is being reevaluated. The central issues in the argument concerns the metabolism of TRI in species which demonstrate hepatocarcinogenicity (mice), species which demonstrate nephrocarcinogenicity (rats), and species which do not show an incidence of toxicity (human) following TRI exposures, and the documentation of human exposures to TRI. A caveat in the human epidemiology studies to date is that human exposures may have involved TRI quantities which would produce toxicity in rodents. The metabolism of TRI involves an initial P450 mediated formation of CH (Byington and Leibman, 1965; Leibman, 1965) and cytochrome

P450 CYP2E1 is the form likely responsible for high rates of CH formation at low TRI concentrations (Guengerich *et al.*, 1991; Nakajima *et al.*, 1990).

Chloral hydrate is further metabolized to TCOH and trichloroacetic acid (TCA). Both TCOH and TCA arise from the metabolism of TRI in the rodent (Larson and Bull, 1992) and from CH in the human (Cabana and Gessner, 1970; Sellers *et al.*, 1972). Further, TRI metabolites CH, TCA and dichloroacetic acid (DCA) have been investigated as mediators of TRI toxicity. Chang and coworkers (Chang *et al.*, 1992) report that TCA and DCA induced strand breaks in hepatic DNA of mice but not rats; while Nelson and Bull (1988) report DNA strand breaks are induced in rats and mice by TRI, CH, TCA and TCOH. Lash *et al.* (1995) have also demonstrated that TRI, CH, TCOH, DCA, and dichlorovinyl cysteine (DCVC) inhibit mitochondrial respiration. Exposure of isolated hepatocytes of rat and mouse to TRI (Klaunig *et al.*, 1989) demonstrated that TRI and the TCA metabolite both produce alterations in mouse, but not rat hepatocyte intercellular communication; and pretreatment with an inhibitor of cytochrome P450 (which acts to decrease conversion of TRI to TCA) minimizes the effect. Studies performed by Miller and Guengerich (1983) found that metabolism of TRI (as measured by DNA- and protein-adducts, chloral and TRI production) varied greatly between mouse, rat and human microsomes; mouse microsomes had highest activities, followed by rat and human and activities were highly variable among the four human samples tested.

It is crucial that in the absence of strict human data, a model species deemed relevant from the standpoints of molecular mechanism of toxicity and TRI metabolism be identified in which to evaluate TRI toxicity. For the above reasons, the rat and mouse may be inappropriate. Because metabolism modulates TRI toxicity, the data used to produce a human health risk assessment should be derived from species which metabolize TRI in a manner similar to that in humans. No epidemiology study of humans in the current literature has revealed a link between TRI exposure and carcinogenicity. Because rodents possess a higher metabolic capacity than humans, toxicity assessments based solely upon data derived from rodents may over-predict the human response. In addition, at least one TRI metabolite has been identified in rodents which does not seem to be identifiable in humans. Such qualitative and quantitative differences reduce the value of rodent toxicity data. The exposure of high numbers of rodents to very low doses of TRI is necessary to determine the response at low levels of exposure, as might occur in humans. The identification of a species which can be exposed to TRI in a cost-effective manner and which metabolizes TRI in a manner similar to that observed in humans will lower the costs associated with development of toxicity data from the lower end of the dose-response curve. We have evaluated the qualitative and quantitative metabolism of TRI in the Japanese medaka minnow, a species which may be exposed to TRI in large numbers at low cost. Because of the dependence of TRI toxicity upon metabolism, a metabolic assessment of a proposed species should be the first step in the evaluation of TRI toxicity.

EXPERIMENTAL METHODS

Animals and Tissue Preparation: Male and female Japanese medaka minnows (*Oryzias latipes*) were a generous gift from the Gulf Coast Research Laboratory, Ocean Springs, MS. Animals were euthanized by placing them into water containing 0.5% tricaine methyl sulfate. Their livers were immediately removed and placed into ice-cold 0.9% sodium chloride. At five-minute intervals, livers were weighed and homogenized in 2 volumes buffer containing 250 mM potassium phosphate, 250 mM potassium chloride, 10 mM ethylenediaminetetraacetic acid (EDTA), 250 mM phenylmethylsulfonyl fluoride, and 0.1 mM dithiothreitol, pH 7.4. Liver homogenate was centrifuged 20 minutes at 9,000 x g to pellet cellular debris, erythrocytes and nuclei. Resulting supernatant (S_9 fraction) was centrifuged 1 hr at 105,000 x g to sediment the microsomal membrane fraction. The supernatant was removed and the pelleted microsomes were resuspended in a washing buffer containing 200 mM potassium pyrophosphate, 250 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 0.1 mM dithiothreitol, pH 7.4. and were centrifuged as before. The resulting washed, microsomal pellet was resuspended a final time in 0.5 mL of buffer containing 100 mM potassium phosphate, 100 mM EDTA and 20% glycerol, pH 7.4. Microsomal protein was quantified by reaction with bicinchoninic acid (BCA, Pierce, Rockford, IL) and cytochrome P-450 was quantified by differential spectrophotometry with carbon monoxide binding using the method of Omura and Sato, 1964.

Exposure Conditions: Trichloroethylene was dissolved in acetone and injected directly into microsomal suspensions containing 0.1 to 2.0 mg microsomal protein per mL, 3 units glucose-6-phosphate dehydrogenase, 13 micromoles glucose-6-phosphate and 0.9 micromoles NADP in 0.1 M TRIS/5 mM magnesium chloride. Final concentration of acetone in these incubations was 0.1%, which was shown not to inhibit P450 1A-dependent reactions (not shown). Chloral hydrate was dissolved in 0.1 M TRIS/ 5 mM magnesium chloride and diluted into a system including 0.1 to 5 mg S_9 protein/mL and 0.9 micromoles cofactor [nicotinamide adenine dinucleotide (NAD); nicotinamide adenine dinuceotide, reduced form (NADH); nicotinamide adenine dinucleotide phosphate (NADP); or nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)] per mL. Following incubation, CH was extracted from TRI-exposed microsomes by the addition of 400 µL incubate to a vial containing 800 µL ethyl acetate and extracted for 20 minutes at 37°C. Trichloroethanol was extracted from chloral hydrate-exposed S_9 protein under the same conditions. Trichloroacetic and dichloroacetic acids were extracted from chloral hydrate-exposed S_9 protein by the method of Maiorino *et al.*, 1980. Briefly, 100 µL of incubate was acidified with sulfuric acid then TCA and DCA were derivitized with dimethyl sulfate and extracted into hexane for analysis.

Mass Spectral Confirmation of Chloral Hydrate: Medaka microsomal protein and NADPH were incubated in TRIS buffer in the presence and absence of trichloroethylene for 30 minutes and extracted with 1 volume ethyl acetate. Ethyl acetate extract was concentrated to virtual dryness under a stream of nitrogen and 20 µL of hexane were added to dissolve the residue. Authentic chloral hydrate was dissolved in hexane. Both hexane solutions were analyzed by gas chromatography-mass selective detection. One-microliter volumes were manually injected onto a Hewlett-Packard Model 5890A Series II gas chromatograph equipped with a DB™-624

capillary column (0.25 mm X 30 m, 1.4 um film thickness). Injector temperature was 175°C, oven temperature was held at 50°C for 8 minutes and increased 10°C per minute to 175°C and held there for one minute. Library data for standard compounds were searched and used to determine which range of atomic mass units to monitor for chloral hydrate detection. Detection was accomplished via a Hewlett-Packard Mass Selective Detector monitoring ions of 47, 82, 84 and 111 AMU.

Quantification of Chloral Hydrate: Chloral hydrate and trichloroethanol were separated and identified by gas chromatography-electron capture detection. One microliter liquid injections of ethyl acetate extract were made by a Hewlett-Packard 7673 injector onto a VoCol™ capillary column (0.53 mm X 30 m) in a Hewlett-Packard 5890 gas chromatograph. Initial oven temperature was 60° for 5 minutes, then increased by 15°/min to 90° and held for 3 minutes. Temperature was then decreased by 15°/minute to 60° and held until the next injection. Argon/methan (95/5) flow rate was 30 ml/min and TRI, CH and TCOH retention times were approximately 3.0, 3.6, and 8.2 minutes, respectively. Injector temperature was 175°C and detector temperature was 230°C. GC signals were collected and integrated by a PE-Nelson TurboChrom 4 data acquisition system. Chloral hydrate and trichloroethanol were quantified against a standard curve of authentic compound dissolved in ethyl acetate. Separate experiments determined extraction efficiency from biological matrix. Trichloroacetic acid-dimethyl sulfate complex was injected in 1 microliter hexane extract onto a DB™-Wax capillary column (0.53 mm X 30 m). Detection was by ^{63}Ni electron capture detection system and signal was captured and processed as above.

Electrophoresis of Proteins and Western Blot Analyses: Ten picomoles of P450 from human, rat and mouse liver microsomes (except 5.12 pmol from female medaka) were separated by electrophoresis on 12% SDS-polyacrylamide gels as described by Laemmli (1970) and electroblotted onto nitrocellulose membranes. Conditions of Western blot analysis were as described by Towbin *et al.* (1979). The immunodetection of protein bands on the nitrocellulose membranes was carried out as described previously using the appropriate primary antibodies to different P450 forms and secondary antibodies conjugated to alkaline phosphatase.

RESULTS

Demonstration of Chloral Hydrate as a Trichloroethylene Metabolite in Medaka Microsomes: Metabolism of trichloroethylene to chloral hydrate was confirmed by the analysis of metabolite extracted from incubations containing TRI, medaka microsomes and NADPH. No metabolite was identified in incubations lacking only NADPH and no metabolite was identified in incubations lacking only protein. Gas chromatography - mass spectral identification of chloral hydrate is presented in *Figure 1*: metabolite extracted from medaka microsomal incubations is represented in panel A, authentic chloral hydrate is in panel B, and the library match for trichloroacetaldehyde is in panel C. Analysis of authentic chloral hydrate identified the most abundant ions as the 47, 82/84 and 111 AMU fragments, in agreement with the library standard for chloral hydrate (trichloroacetaldehyde). The ions of 111, at 82 and 47 mass units represent fragments of CCl_2-COH, C-Cl_2, and CCl ions, respectively. Analysis of the experimental extract revealed agreement among metabolite isolated from TRI-exposed medaka microsomes, authentic chloral hydrate and the library standard. No similar compound was identified in naive medaka microsomes or in incubations containing trichloroethylene but no microsomal protein.

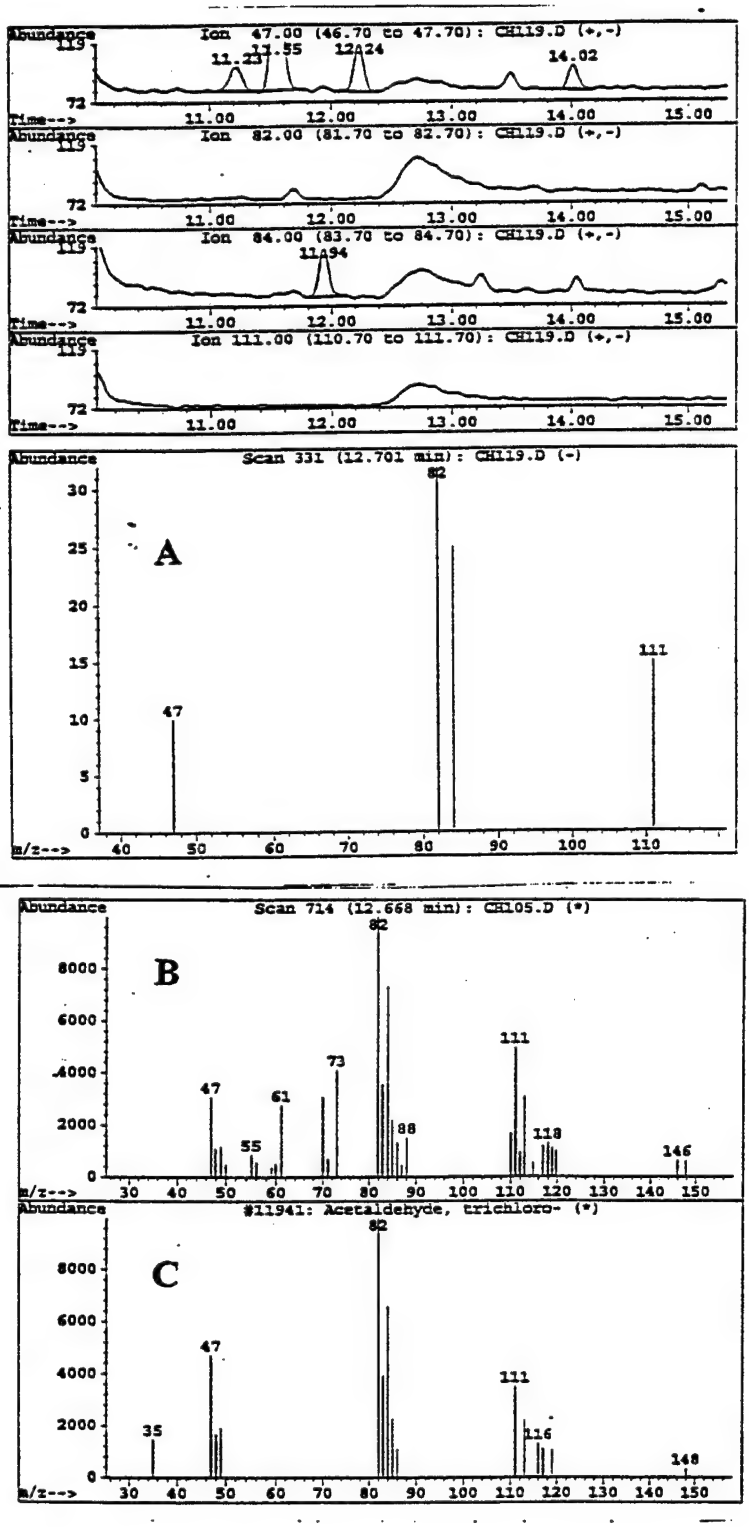

FIGURE 1. Gas Chromatography/Selected Ion Monitoring Confirmation of Chloral Hydrate. Metabolite extracted from medaka microsomal incubation with NADPH and TRI (panel A) is compared to authentic chloral hydrate (panel B) and library standard for chloral hydrate (trichloroacetaldehyde, panel C). Results confirm chloral hydrate as a metabolite of TRI in medaka microsomes.

Demonstration of Protein and Time Dependence of the Reaction: The further examination of TRI metabolism was accomplished by determining the time and protein constraints of the reaction. Results presented in *Figure 2* indicate that the reaction remained linear to one hour and to a protein concentration of 1.0 mg microsomal protein/ml. We used these parameters to determine the kinetics of the reaction in microsomes.

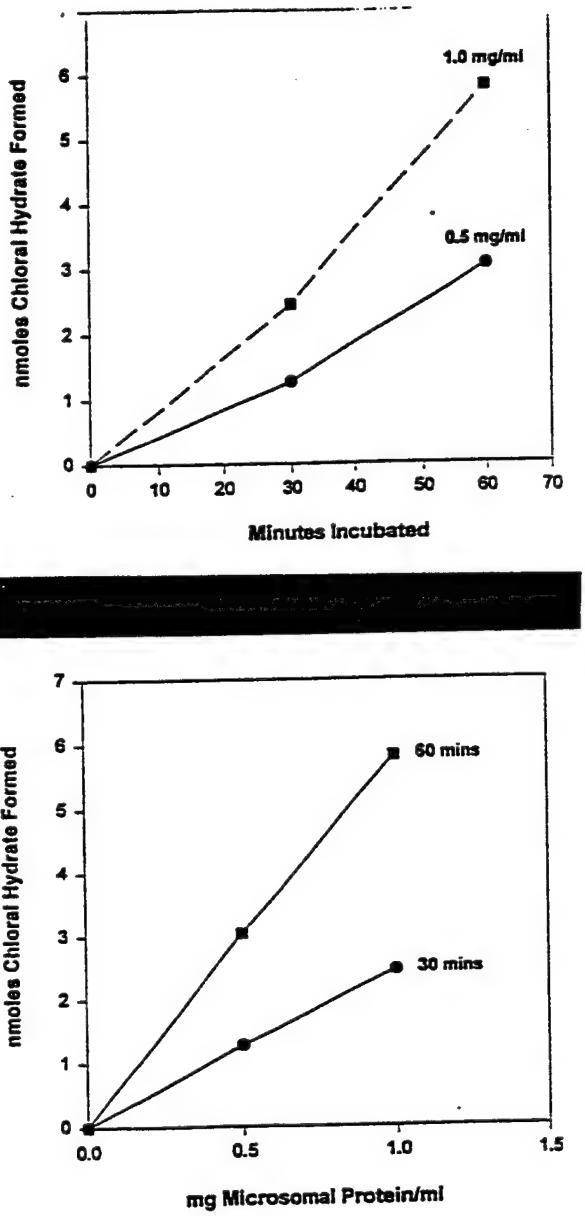

FIGURE 2. Time and Protein Dependence of the Reaction. Chloral hydrate formation from TRI was monitored by GC-ECD at 30 and 60 minutes with either 0.5 or 1.0 mg microsomal protein present per mL. Results indicate that the reaction rate is linear over one hour and to a protein concentration of 1.0 mg/mL.

Kinetics of formation of Chloral Hydrate from Trichloroethylene: Results presented in *Figure 3* indicate saturation of metabolism at high concentrations of TRI. Michaelis-Menten analysis of data indicate a K_m of 540 μM TRI and a V_{max} of 213 pmol/min/mg protein.

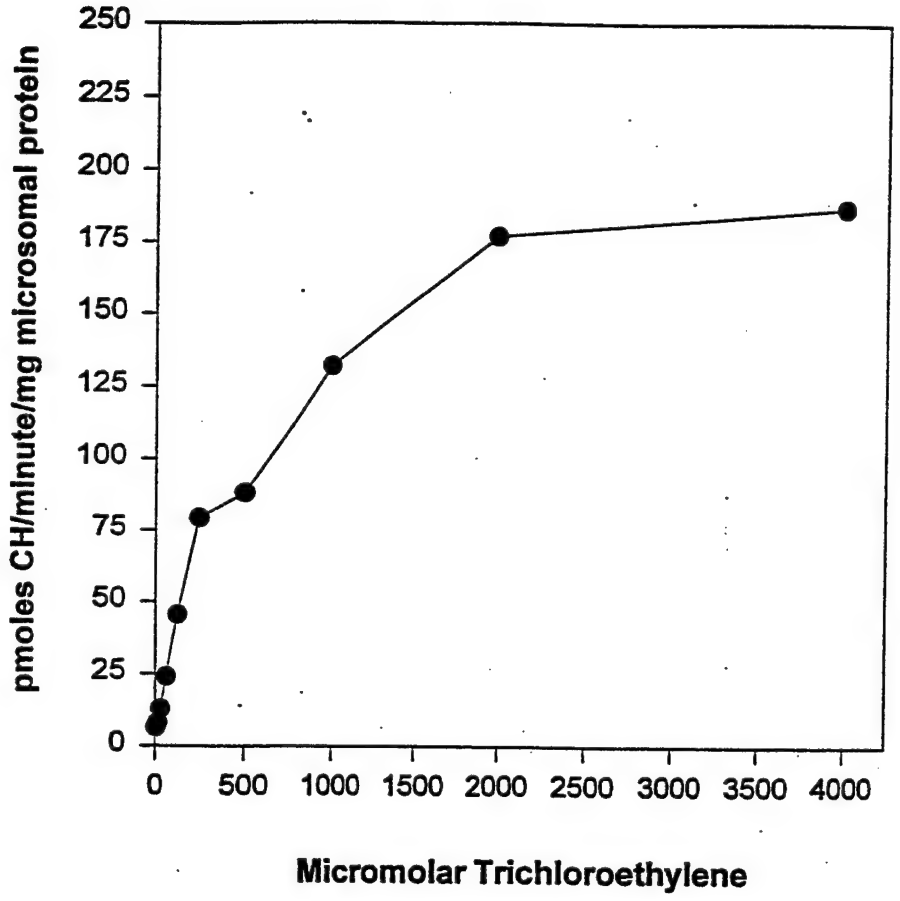

FIGURE 3. Kinetics of the Chloral Hydrate Production. Medaka microsomes were incubated with increasing concentrations of TRI with NADPH for one hour. Production of CH became saturated at high levels of TRI. Kinetic analysis indicated a K_m of 540 μM TRI and a V_{max} of 213 pmoles/min/mg microsomal protein.

Evaluation of Sex-Differences in Rates of Biotransformation: Because of the casual observation of differences in gross liver sizes between male and female medaka, we sought to determine whether this had any potential impact on metabolic capacity between the genders. Recovery of total liver mass, metabolically active protein, cytochrome P-450 and a marker for cytochrome P-340 1A activity was assessed in pools of livers from male and female medaka. Results presented in *Table 1* indicate that the liver mass is larger in female fish, but that total metabolic activity (product formed per liver) is compensated for by a generally higher specific activity (product per unit protein) in male fish.

TABLE 1. COMPARISON OF SELECTED HEPATIC METABOLIC PARAMETERS BETWEEN MALE AND FEMALE MEDAKA

Parameter	MALE	FEMALE
Liver Mass (mg)	12.44	18.93
S_9 Protein/liver (mg)	0.33	0.54
Cytochrome P-450:		
-nmol/mg S_9	0.311	0.245
-nmol/g liver	8.37	7.04
-nmol/liver	0.104	0.133
EROD Activity:		
-pmol/min/mg S_9	0.176	0.128
-pmol/min/nmol P-450	0.565	0.521
-pmol/min/liver	0.059	0.070

Male (n = 147) and female (n = 112) medaka were euthanized, weighed, their livers removed, weighed, homogenized and centrifuged to yield S_9 fraction. Cytochrome P-450 analysis was performed by carbon monoxide spectral shift and activity towards ethoxyresorufin (EROD), a marker for CYP1A, was assessed by spectrofluorimetry.

The evaluation of TRI metabolism in male and female medaka (*Figure 4*) verified this finding. A further assessment of TRI metabolism involved the identification of chloral hydrate-derived metabolites. The formation of trichloroethanol (TCOH) and trichloroacetic acid (TCA) was determined in hepatic S_9 from male and female medaka.

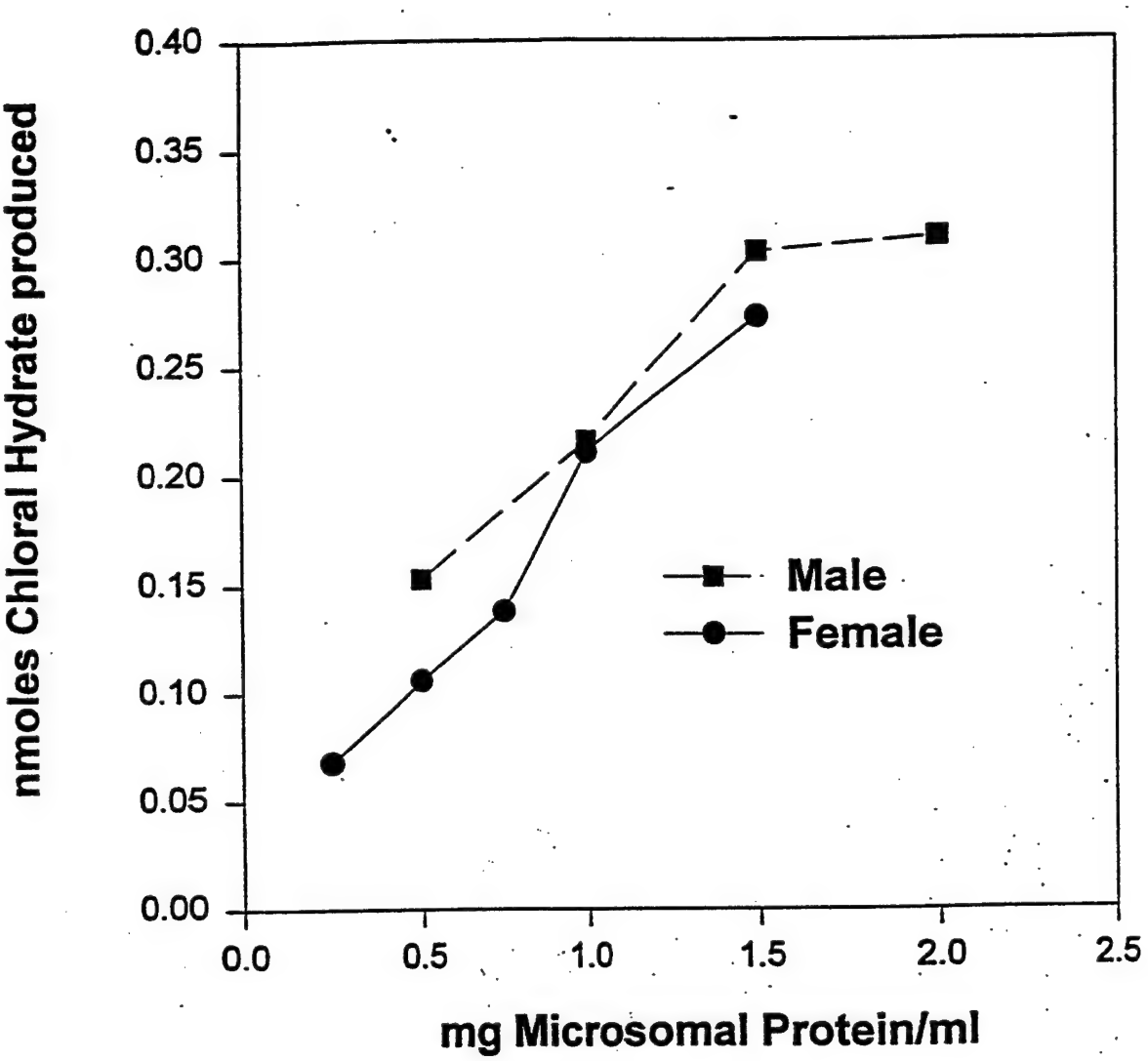

FIGURE 4. Sex Dependent Metabolism of TRI to CH. Microsomes of male and female medaka were incubated with 100 μM TRI for 30 minutes and CH formation was quantified by GC-ECD. Results indicate that microsomes from male medaka possess a slightly higher intrinsic activity towards TRI than do microsomes from female medaka. This result is compensated by a larger liver in female fish.

Results presented in *Table 2* further indicate a higher specific activity in male fish, and indicate that TCOH is the predominant hepatic metabolite of CH.

TABLE 2. METABOLISM OF CHLORAL HYDRATE BY HEPATIC S_9 FRACTION FROM MALE AND FEMALE MEDAKA

	MALE		FEMALE	
Treatment	TCOH	TCA	TCOH	TCA
Control	n.d., n.d.	n.d., n.d.	n.d., n.d.	n.d., n.d.
NAD	23.36, 20.95	n.d., n.d.	15.13, 16.26	n.d., n.d.
NADH	1.54, 1.47	n.d., n.d.	1.07, 1.00	n.d., n.d.
NADP	14.05, 12.45	1.47, 1.53	5.35, 5.62	1.04, 0.98
NADPH	1.61, 1.34	n.d., n.d.	0.80, 0.80	n.d., n.d.

Data are presented as nmoles product formed from the incubation of 3 mg S_9 protein/mL for one hour with 100 uM chloral hydrate and 0.88 mM pyridine cofactors present. Control group had no cofactor added. n.d. = no product detected.

Immunochemical Probing of Microsomes for Cytochrome P-450 forms: Results from immunochemical detection of medaka cytochrome P-450 forms support out lack of demonstration of CYP2E1-dependent activity in medaka. Immunoblots in *Figure 5(A)* indicate that polyclonal anti-rat CYP2E1 IgG does not recognize microsomal proteins from medaka (lanes 17 - male and 18 - female) but recognizes proteins from rat (lanes 2, 13, 14, and 19), mouse (lanes 15 and 16) and humans (lanes 3-12). Lanes 1 and 20 were intentionally left blank. When the same microsomal samples were probed with a polyclonal antibody raised against a synthetic peptide derived from trout cytochrome P-450 1A1 (*Figure 5B*), a different response was noted. This antibody recognized microsomal proteins of medaka (lanes 17 - male and 18 - female), rat (lanes 15 and 16), mouse (lanes 19 and 20), and humans (lanes 5-14). Lanes 1-4 represent purified rat CYP1A1 at 0.5, 0.25, 0.10 and 0.05 pmol/lane, respectively.

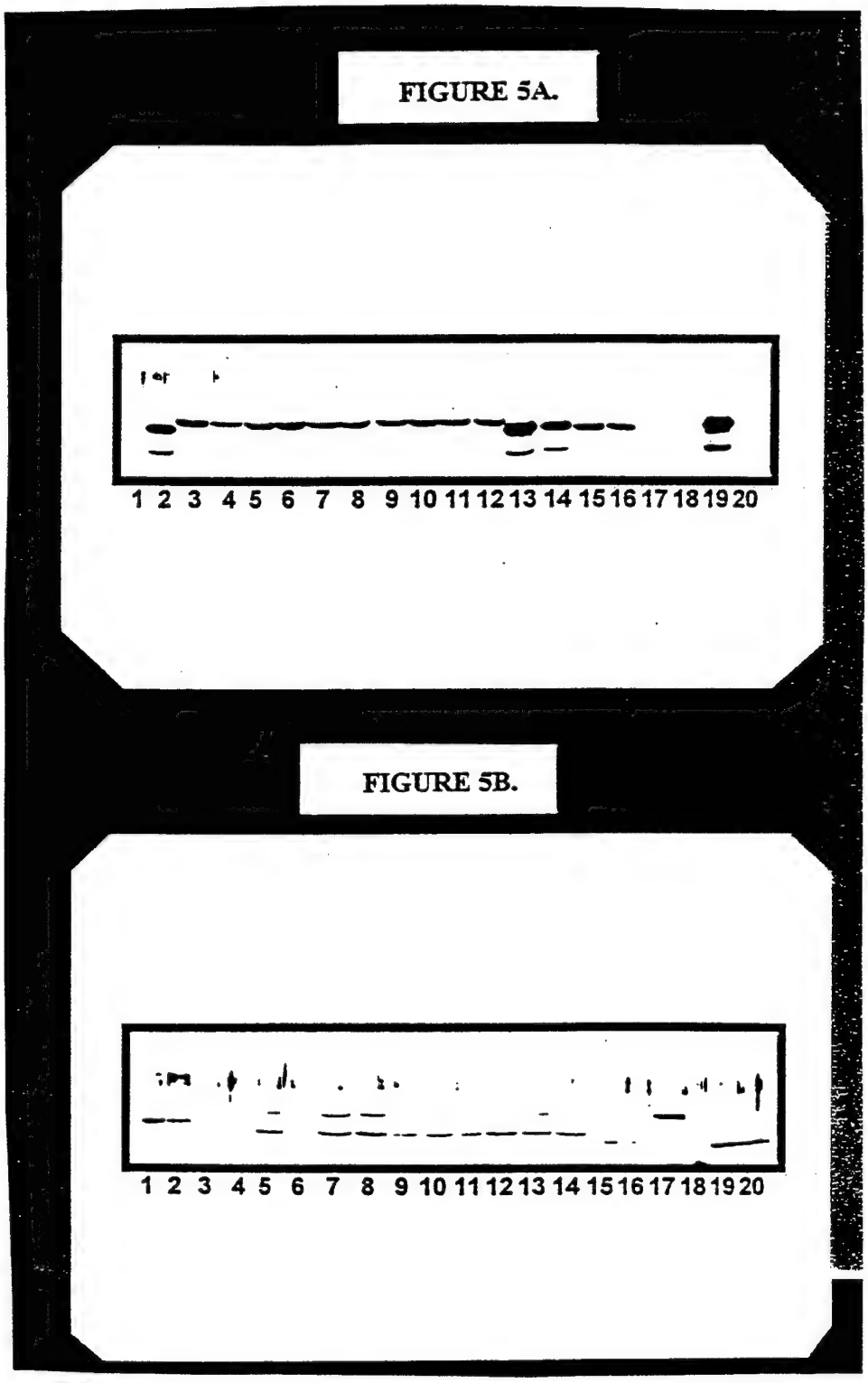

FIGURE 5. Immunochemical Detection of CYP2E1 and CYP1A1 in microsomes of rats, mice, humans and medaka. Results indicate that microsomes from male and female medaka do not display detectable quantities of CYP2E1 (Panel A), but do express CYP1A1 protein (Panel B).

DISCUSSION

The present understanding of trichloroethylene-induced toxicity (NCI, 1976; NTP, 1983; NTP, 1987) in the mammalian systems includes the mediation of toxicity by metabolites of trichloroethylene: both TCA and DCA induced peroxisomal proliferation (DeAngelo *et al.*, 1989; Nelson *et al.*, 1989) and liver tumors (Daniel *et al.*, 1992; Herren-Freund *et al.*, 1987) in lab mammals. The formation of these metabolites is dependent upon the initial action of cytochrome P450 (Byington and Leibman, 1965; Ikeda *et al.*, 1980; Liebman, 1965). Published reports indicate that cytochrome P450 2E1 (CYP2E1) is the form primarily responsible for the formation of CH from TRI in the rat and mouse (Nakajima *et al.*, 1988, 1990, 1992). Data also support the contribution of other P450s including CYP1A forms (Nakajima *et al.*, 1992).

In contrast to the mammalian liver, the liver of fish possess no immunologically-detectable CYP2E1 and no detectable activity towards substrates which are selectively metabolized by CYP2E1 in the mammal. Our experiments here demonstrate the same conclusions in the medaka: antibody to CYP2E1 did not react with microsomal protein isolated from medaka liver and we could not demonstrate activity towards dimethylnitrosamine, a substrate selectively metabolized by CYP2E1 in rats, mice and humans. In addition, no immunologically-detectable P450 3A isoforms were detected in medaka microsomes and we also failed to demonstrate activity towards erythromycin, a marker substrate for CYP3A activity in mammals. We did, however, demonstrate that medaka microsomes metabolize trichloroethylene to chloral hydrate. Significant amounts of immunologically-detectable CYP1A and activity towards ethoxyresorufin, a marker substrate for CYP1A activity in mammals. The immunological detection of CYP1A was stronger in microsomes from male medaka than from female.

The finding of TRI metabolism in a species evidently devoid of CYP2E1 further implicates other forms in this process. Nakajima *et al.* (1993) has demonstrated that antibody to CYP1A reduces the metabolism of TRI as catalyzed by hepatic microsomes. The predominance of CYP1A forms in medaka (and fish, in general) and the conclusive demonstration of TRI metabolism in medaka *in vitro* indicates that CYP1A forms play a role in TRI metabolism. Although CYP2E1 likely metabolizes TRI at high rates at low substrate concentrations (Km ~ 25 μM), CYP1A becomes metabolically active at higher TRI concentrations. Unpublished reports (Lipscomb *et al.*, 1996) indicate that CYP2E1 accounts for 60-85% of TRI metabolism associated with human microsomes and that the V_{max} in the human is approximately 1440 pmol/min/mg microsomal protein. A comparison with TRI metabolism in medaka (where CYP1A is the predominant P450 form) indicates that CYP1A is much less active towards TRI.

Because of the dependence of TRI's metabolism upon toxicity, the assessment of TRI metabolism should be a critical early point in the investigation of possible animal models in which to examine TRI's toxicity. As in the mammal, we have demonstrated the ability of medaka liver supernatant to form TCA and TCOH from chloral hydrate. The formation of TCOH predominates TCA formation, and is stimulated best by NAD, the cofactor which best stimulates TCOH formation in rat, mouse and human liver supernatant. As in the mammalian system *in vitro*, no dichloroacetic acid was detected in medaka samples. All these data indicate

that the medaka is metabolically competent to dispose of trichloroethylene in a manner similar to the human and laboratory rodent. These data indicate that the medaka may be a logical choice for an experimental model. Medaka are inexpensive to raise and can be exposed in simple aquaria to very low concentrations of TRI in large numbers. These conditions may favor the detection of low incidences of toxicity, as would be predicted to occur at the lowest end of a dose-response curve.

CONCLUSIONS

- Medaka have no detectable CYP2E1
- Medaka microsomes metabolize TRI to CH
- Rates of CH formation by medaka microsomes are 200 pmol/min/mg, while rates in the human, rat and mouse are 1440, 4826 and 5425 pmol/min/mg, respectively
- Medaka S_9 metabolizes CH to TCOH and TCA
- No DCA was detected in medaka samples *in vitro,* likewise in mammalian samples
- Differences in the specific metabolic activity between sexes is compensated by liver size and protein content
- CYP1A1 metabolizes TRI to CH
- The medaka is similar to the mammal in qualitative TRI metabolism
- Medaka may be a suitable species in which to monitor TRI toxicity

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